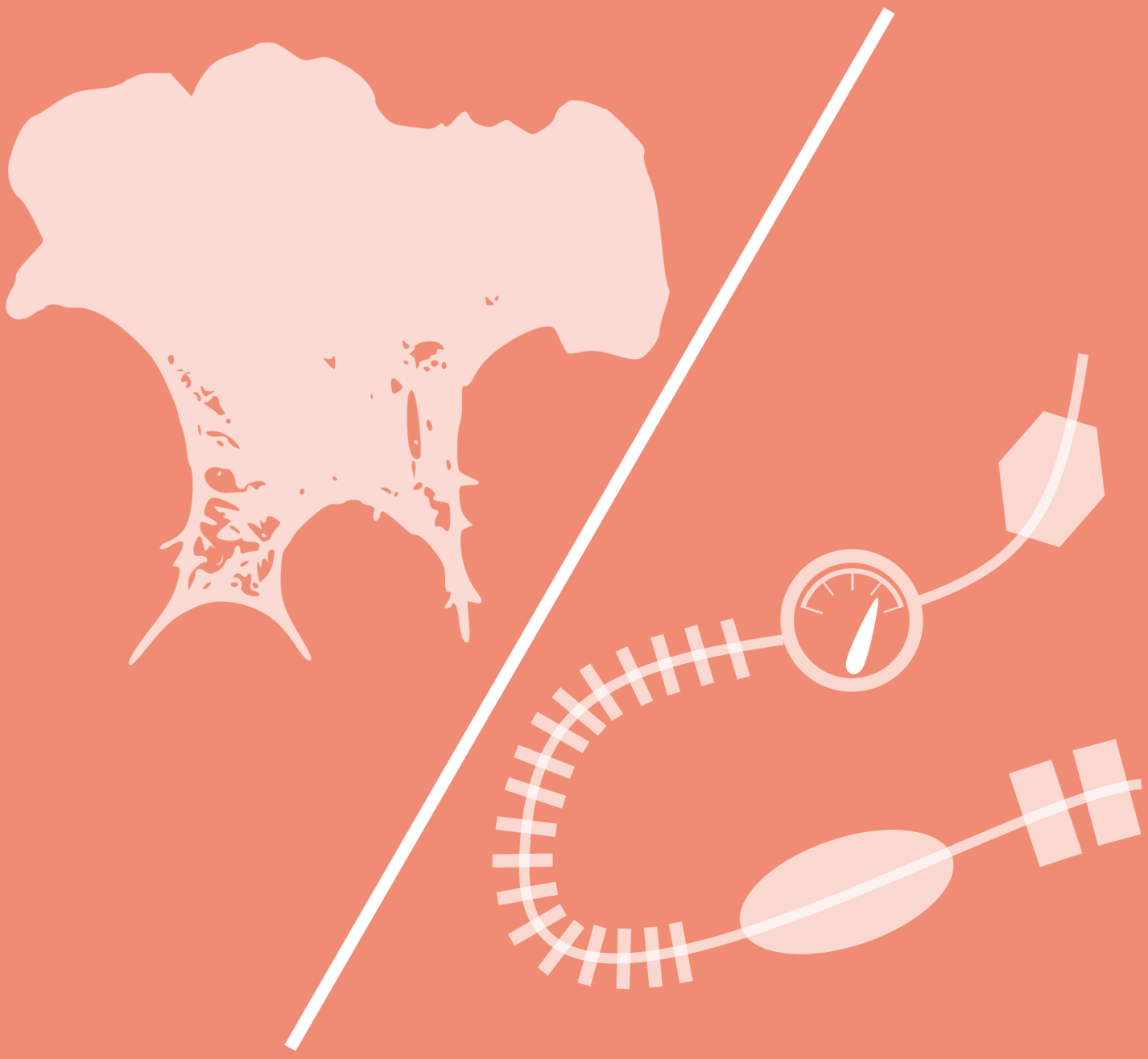

18

Nemo Andrea

Cell Migration, Molecular Clutch and a force-brearing role for Spectraplakins

Research Proposal for
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TU Delft

Research Proposal

Proposing a novel platform to study the dynamics of Amoeboid to mesenchymal Migration, molecular clutch engagement and cytoskeletal dynamics.

Nemo Andrea

In this research proposal, we propose the design of a novel platform to study a cellular process known as the Amoeboid- to Mesenchymal migration Transition (AMT). This switching of migratory modes is observed in various cell types, and plays a central role in the metastatic processes in cancer. Previous experiments have shown it is possible to induce AMT, and its counterpart MAT, by inhibiting factors known to be central to cell migration. At the current time, there is no standard platform to study the dynamics of these transitions. Here, we describe a design for a platform that would allow for a standardised method to study AMT/MAT. Furthermore, we list examples for new research question that this platform could greatly aid in addressing. Besides validation experiments for the development of this platform, we also suggest design of FRET experiments involving spectraplakins, large cytoskeleton crosslinking proteins that have recently been associated with human disease and cell migration. With these two topics, we both hope to answer how cells transition through geometrical constraints and to answer what the forces across spectraplakins are and how these forces are related to its functioning.

1 Background

The ability to migrate to different regions is essential for life, as it allows for the organism to move to a more favourable environment. It is therefore no surprise many cells possess the ability to migrate. The two predominant forms of migration are flagellar-based or crawling-based. In animals, nearly all cells migrate using the crawling principle - from now on simply referred to as migration, with a notable exception being swimming sperm. While not all cells migrate frequently, there are many cell types that do migrate, and whose deficiency in migration is associated with disease.[1] Macrophages and Neutrophils are immune cells that actively migrate, using an amoeboid form of migration. Osteoclasts and Fibroblasts are other examples of frequent migrating cells, whose deregulation is associated with disease.[2] Epithelial exhibit perhaps the most spectacular migration, often migrating collectively in sheets. [3][4] It is therefore of great interest to understand the detailed functioning of this crawling-based movement, as is a mechanism employed by cells in the human body.

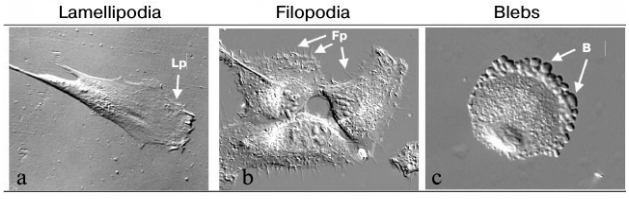

Figure 1: Figure taken from (Alexandrova 2014)[5]; crawling migration: left and middle; amoeboid migration: right

It was realised early on that the underlying mechanism for migration, is primarily driven by actin polymerisation. In brief, migration can be divided into a few distinct processes that must all occur if a cell is to migrate effectively. (1) Polarisation, where the cell rearranges its interior complexes to induce a certain directionality to the cell. Maintaining a polarisation will allow a cell to continuously migrate in one direction.[6] (2) Protrusion, where protrusions occur in the membrane and, depending on the type of migration, extensions form (lamellipodia or filopodia), or blebs form (blebbing migration)[7]. These protrusions occur in the direction of polarisation. (3) In order to move, a cell must be able to exert traction on the environment.

In mesenchymal migration (utilising filopodia and lamellipodia), this is achieved by physically linking the internal cytoskeleton to the extracellular matrix (ECM). In order to move, it is critical to have connections be made only at the ''front" of the cell, and have connections be disengaged at the cell rear. In amoeboid (blebbing) migration, cells exert traction by contracting and expanding. The force required for cell migration is generated by actin polymerisation. While the exact mechanism and interplay of regulatory molecules of the actin cytoskeleton are not known, it is clear that the nucleation of new actin on existing actin filaments that are linked to the ECM through complicated structures, provides the protrusive, and thereby the motive force.[8]

These three processes must all be tuned correctly in order for the cell to move, and disruption of any of the three will affect cell migration. The ability of cells to sense the mechanical properties of the environment is known as mechanosensing, and allows cells to tune the forces and speed of migration. The component that links the ECM to the (actin) cytoskeleton is known as the molecular clutch.[9] This model revolves around, as mentioned earlier, actin polymerisation generating the force for migration, but is not always coupled to the ECM. The cell can selectively regulate the linking of the integrins (proteins connecting ECM and cell) to the polymerising actin. To go

with the clutch analogy, the actin would represent the engine, the integrins linked to the ECM the wheels, and the Focal adhesion complexes would be the clutch. As the Focal adhesions link the actin to the integrins, forces generated by polymerisation gets transferred onto the integrins and the cell is pushed forwards. As actin can be linked to many different contact points (as well as the possibility of molecular slippage), there is a degree of tunability to clutch engagement, allowing for precise control of migration speed.

A majority of the historical work on cell migration and its regulation has been conducted on cells migrating on flat surfaces, which, essentially, limits cell migration to 2D.[10] While these experiments have greatly expanded our knowledge of migration and the factors regulating it, it is apparent that certain effects observed in 2D do not occur in 3D. Recent research of cell migration is therefore focused on cells migrating in 3D.[11] The technique we propose here aims to make use of cells mechanosensitive response in a 3D environment, thus facilitating research of the more physiologically relevant 3D cell migration.

1.1 Involved Signalling

As mentioned before, the regulation of cell migration is highly complex, with many regulatory proteins and factors having been uncovered.[12][13] Here we will list a few of these proteins, to provide a better idea of the composition and regulation of migration.

Focal adhesions (FA) are multi-protein structures that are essential for mesenchymal migration, functioning as anchor points for the cell. They link the cytoskeletons to the ECM. Similar to cell migration as a whole, they are highly dynamic and undergo various phases. These structures are often referred to as the feet (or of the cells, as parallels can be seen in their function. FAs can grow in size and protein composition and can also be dissembled. It is well established that these states are spatiotemporally regulated, with FAs responding to mechanical, biochemical and structural cues.[14]

A recent study has uncovered the highest z-resolution image/model of FAs to date.[15] It was shown that the many proteins involved in focal adhesion are present in distinct distances from the membrane (z-height). Focal adhesions thus have a distinct 3D composition. This result was obtained by studying cells migrating in 2D, and whether the exact distances hold for 3D migration is not known. Recent developments in imaging such as STORM will allow for the study of FAs at unprecedented resolution.[16]

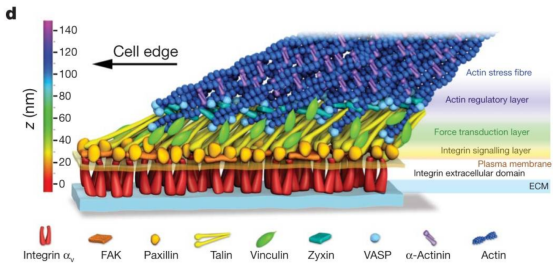

Figure 2: Image taken from [15]. Schematic model of focal adhesion molecular architecture, depicting experimentally determined protein positions. Note that the model does not depict protein stoichiometry.

Integrins are a family of transmembrane proteins that are essential for a type of cell movement known as integrin-dependent (mesenchymal). They can aggregate in membrane locations, leading to the formation of precursors of focal adhesions called nascent adhesions.[14] in line with their central role in mechanosensing, integrins can respond to mechanical stretching by changing conformation. A work by (Chen et al. 2012)[17] suggested a model wherein the stretching of integrins induced a conformational change which increases affinity for ECM binding. Integrins serve as the main transmembrane protein in cell migration, serving as both an active component in mechanosensing and forming the structural element linking the internal focal adhesion structure and ECM.[18]

In order for the forces generated by actin to be transferred to the ECM through integrins, a mechanical connection between actin and integrins must be established. One of the proteins involved in this connection is talin. Similar to integrins, forces across vinculin can induce a conformational change and, in talin's case, expose hidden binding sites.[19] Talin can directly bind to integrin and actin.[20]

Vinculin is another important protein in focal adhesions, being critical for both formation and stabilization. It has been found to affect paxillin, the ERK1/2 pathway, and the FAK-paxillin interaction.[21] While vinculin is thought to not be mechanosensitive itself, vinculin levels have been found to associate linearly with traction forces.[22]

Focal Adhesion Kinase (FAK) is a protein that interacts with many different proteins and is, as the name suggests, closely involved with Focal Adhesions. It is involved in many pathways, with various efforts undertaken to elucidate its functions.[23][24] A recent work by (Ritt et al. 2013)[25] used a new FRET based technique to characterize the activity and spatial patterning of FAK.

Rho family GTPases play an important role in cell migration, with RhoA, Rac1, and Cdc42 controlling various aspects of both amoeboid and mesenchymal migration. Rho GTPases function as molecular switches by using guanine nucleotide-exchange factors (GEFs).[26]

RhoA is an important regulator of actomyosin contractility, and actin itself, closely interacts with- and activates ROCK, and stress fiber formation.[27] Rac and Cdc42 stimulate the formation of lamellipodia and filopodia, a signature of mesenchymal migration. As these Rho GTPases regulate many different aspects of migration, their role is hard to define for a cell as a whole and should ideally be discussed only in the context of their cellular location.[28]

RhoA and RAC1 are antagonistic, resulting in them often being considered to be one of the important determinants of cell migration mode.[29] Recent works have revealed that the Rho GTPases and their contextual regulation exhibits and 'unanticipated degree of complexity'.[30]

In order to navigate the dense and crosslinked ECM, cells can use metalloproteinases (MMPs) to degrade the ECM locally. Cells have been known to migrate both using MMPs and without.[31] The activity of MMPs is also dependent on the adhesion, force generation, and rigidity sensing of migrating cells.[32] The ability of cells to directly modify and break down the surrounding structure provides an alternative to migrating around structures or trying to ''squeeze through" using amoeboid migration.

1.2 Cytoskeleton

Besides single proteins or factors, cytoskeletal components also play key roles in cell migration. As mentioned before, the actin cytoskeleton is core to cell migration, but it is becoming increasingly apparent that the microtubule cytoskeleton also plays a big part in the regulation of migration.[33]

Actin is a highly conserved and abundant protein. Various isoforms exist, each expressed in specific tissues.[34] One of the key features of actin is its ability to interact with itself. Through this self-interaction it can form filaments (i.e. polymers). The monomeric form is known as G-actin, the filaments known as F-actin. The filaments are linear and double stranded, with a persistence length of around 17 μm .[35] Due to its abundance and mechanical properties, actin is very important for the structural support of cells. The continuous (de)polymerisation of actin can also generate forces through a ratcheting mechanism.[36] Additionally, a motor protein called myosin can bind to two actin fibres and cause these to slide past each other, allowing for generation of contractile forces. A specific organisation of actin is known as the cortical actin cytoskeleton. This network, as the name implies, lies in the cell cortex just below the membrane. Here actin is connected to the membrane through membrane-microfilament binding proteins.[37] This structure is partially responsible for cell shape and is under tension, allowing cells to resist compressive forces.[38]

Tubulin is another protein that interacts with itself and forms filaments. Whereas Actin assembles into a double stranded linear filament, tubulin monomers assemble into a hollow cylinder. They are much stiffer than actin, with a persistence length of well over a millimetre.[39] Similarly to actin, the microtubule cytoskeleton is highly dynamic, constantly adding and removing monomers from filaments. In cell migration, the current presumed role for microtubules is as a transport network. Microtubules, however, are able to generate forces themselves, which may turn out to play a role in cell migration, as many of the components in said process are mechanosensitive and may respond to forces generated by microtubules. A recent paper33 mentioned that the potential mechanical role of microtubules in cell migration deserve to be further investigated, particularly the tensegrity model.[40]

α-Actinin is an actin-binding protein and a member of the spectrin superfamily. Different isoforms and genes can be found in mammals, with variation stemming from alternative splicing. At least six different protein forms are known to be present in mammals.[41] α-Actinin features two actin-binding domains (ABD), allowing it to crosslink actin filaments, which is its primary function. The ABD is a highly-conserved region of the protein, while the region linking both ABDs is less conserved, allowing for changes in mechanical properties of the crosslinking function of the protein. α-Actinin has been found to bind directly to the cytoplasmic tails of integrin.[42] Additionally, they were also found to interact with vinculin, further cementing a role for this protein in cell migration. Besides interaction with these cell migration proteins, the ability to crosslink actin and thereby module the geometry of the actin network is also key; as the force generated for migration is through actin. α-Actinin is found in special actin bundles known as stress fibres. These structures can be seen as functional analogues of the Z-disk.[43] Stress fibres are highly crosslinked regions of actin bundles (often) with myosin. These fibres are often connected to Focal Adhesions and can, due to the presence of myosin, contract.[44]

Spectraplakins are a family of large proteins. They are proteins that share features of both the Spectrin and Plakin family of proteins; hence the name Spectraplakins. A key feature of these proteins is their ability to bind both Actin and Microtubule; providing a potential mechanical coupling. The Actin and MT region may be separated as far as 400nm.[45] Besides binding actin and MT, (Hamill et al. 2009)[46] showed that a spectraplakin member is able to directly bind to transmembrane proteins, suggesting a role in cell adhesion. Mammals only have two (currently) known members in this family: BPAG1 (Bullous pemphigoid antigen 1) and MACF1 (also known as ALCF7). In Drosophila, a spectraplakin called shot, is found. These proteins can achieve large variation in function through alternative splicing[47] A more detailed section on mammalian spectraplakins will be provided in a later section.

1.3 Mesenchymal Migration

Mesenchymal migration is a form of single-cell migration. It is characterised by the presence and use of distinct adhesive structures. Mesenchymal migration can be divided into multiple parts that all have to occur in order for cells to migrate effectively. These steps are: protrusion, adhesion formation and retraction.[48]

Mesenchymal migration is a common mode of cell migration exemplified by fibroblasts.[49] As the cells adhere strongly to the ECM, they can also exert large pulling forces on it during migration, an effect exploited by TFM. While one may think that the stronger adhesion to the ECM would lead to faster migration, this is not the case. For mesenchymal cells, the ''sweet spot" in adhesion is found in intermediate adhesion, with too little adhesion not allowing for effective traction, and too much impeding migration.[9]

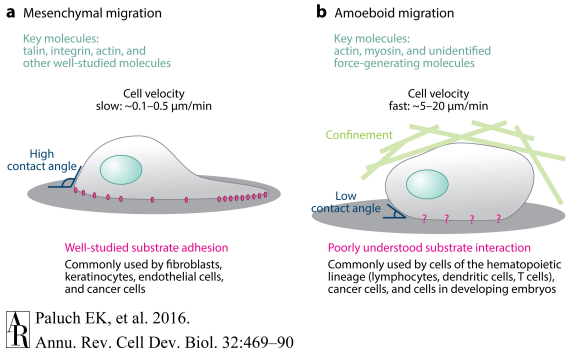

Figure 3: The two primary cell migration modes. Image taken from (Paluch et al. 2016)[50]

1.4 Amoeboid Migration

n contrast to mesenchymal migration, amoeboid migration is a type of migration in 2D and 3D that is characterised by largely integrin-independent motion. The type of motion results in a rounded phenotype. The cells that move by amoeboid mechanisms also do not form focal adhesions during movement.

Cells have been shown to be able to transition between amoeboid and mesenchymal migration, suggesting that the mechanisms are not cell specific, but rather controlled by certain factors that determine migratory mode. Inhibition of various factors has already been shown to be able to induce said transitions.[51] While migration is therefore not cell specific, many cells exhibit only one migratory mode in natural conditions.

Various studies have shown that certain cell types can indeed migrate without integrins, lending to the credibility that the amoeboid migratory mode does not rely on integrins for force generation.[52][53]

While cell migration is faster in amoeboid type migration, this does not mean all cells move in this fashion. The migratory mode depends on various factors, some of which cell intrinsic and some extrinsic.[7] Cortical tension, actomyosin activity[54] and amount of integrin present on the cell membrane are examples of intrinsic factors that can influence which migration mode is used. Extrinsic factors include stiffness and other mechanical properties of the ECM and spatial confinement (see later sections).

Unlike mesenchymal migration, amoeboid migration uses cytoplasmic flow generated by contraction at the rear of the cell to move.

1.5 Hybrid Migration Modes

While these are the two predominant migratory types, certain intermediate types or subtypes can be distinguished. For example, (Liu et al. 2015)[55] recently found that two types of amoeboid cells can be distinguished in a single cell population. There is also variety in the mesenchymal group of migrating cells. We can still maintain the distinction between amoeboid and mesenchymal migration based on cell type, and integrin engagement however.

1.6 Migration in Cancer

Perhaps the most direct medical relevance of research of cell migration can be found in metastasis. Metastases are secondary tumours found in different sites in the body from the primary tumour from which the tumours originate. For tumours to metastasise, tumour cells must be able to migrate from one location to the other. This means that cells experience many obstacles and different (mechanical) microenvironments.

In order to migrate through the large variety of environments, the cells will have to transition to a migratory and invasive phenotype. This often means that the cell morphology will change and adapt to migration and the local environment.[56][57] The degree to which tumour cells can adapt to the environment will depend on the cell type, but certain general patterns can be found. Similarly to what is found in normal macrophages, stiffer ECM will generally result in a preference for mesenchymal migration, whereas a softer ECM will result in a preference for amoeboid migration.[58][59]

As in normal cell migration, cancer cell migration can be divided up into two classes: mesenchymal and amoeboid. And, just like healthy cells, certain cell types are able to transition from one migration mode to the other.[60] This makes the study of migratory mode transitions of high interest to the cancer field, as impairing said transitions is speculated to, in many cases, decrease the effectiveness of cancer cell migration.[61]

Currently, various underlying mechanisms have been identified as playing a role in AMT/MAT. As amoeboid migration relies on (actomyosin) contractility, targeting the Rho/Rock Pathway which normally maintains this contractility, has been shown to

be sufficient to induce AMT.[62] This general process of targeting factors that are known to affect important aspects of either migration type has resulted in many ways to induce AMT/MAT, but only after externally silencing one pathway/factor. In such a way, it was shown that inhibition of RAC, which is important for mesenchymal migration induced MAT-like features.[63] Affecting MT dynamics has also been shown to be able to induce MAT-like features, with stathmin 1 inducing this change. [64]

Finally, modifying the ECM remodelling activity by inhibiting proteolysis pathways of cells has been shown to be enough to induce MAT-like features in fibrosarcoma.[65] This finding suggested that the proteolytic activity of cells is not the crucial determinant of cell invasiveness. As the cytoskeleton is the prime force generating component for migration, it is therefore of tremendous interest regarding metastasis research.[61] An overview of some of the research of migration in cancer lines can be found in (Paňková et al 2010)[51].

Thus, understanding the mechanisms that underlie cell migration transitions will greatly aid us in understanding the process of metastasis, and will allow for new targets for cancer medication.

1.7 State of Field

The expansive characterisation of the many aspects of cell migration has stemmed from and caused the development of many new types of assays for the study of cell migration. These tools have allowed for force quantification inside and outside the cell.[66] These methods all have their unique strengths and weaknesses.

A tool that has seen extensive use in the study of cell migration are micropillars. Micropillars are micromachined cantilevers which are mounted in a horizontal (in line with gravity) fashion, which are mounted in close proximity on a substrate, first used by (Galbraith et al 1997)[67]. Due to the dense packing of micropillars, cells can migrate effectively across them, while the local force exertion can effectively be inferred from the beam deflection-force relationship.[68] The advantages of a micropillar setup is that it one can unambiguously determine the force on each micropillar. This is not always true for other methods. Other advantages are the ease of modification and possibility to detect the (in-plane) direction of the traction force exerted by cells. The downsides are that this method is limited to 2D migration, which, as discussed earlier, is markedly different from more natural 3D migration[11], and the fact that micropillars have a smaller surface/contact area for cells to adhere to compared to a flat surface. An interesting evolution of the passive micropillars is described in [69][70]. In this paper, the authors manufactured the micropillars with magnetic nanowires inside, turning the micropillars into an active force exertion platform.

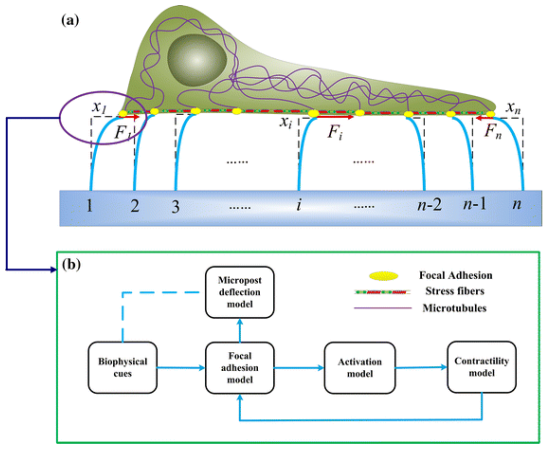

Figure 4: Figure taken from (Geng et al. 2016).[68] Schematic diagram of cellular mechanotransduction on micropost arrays.

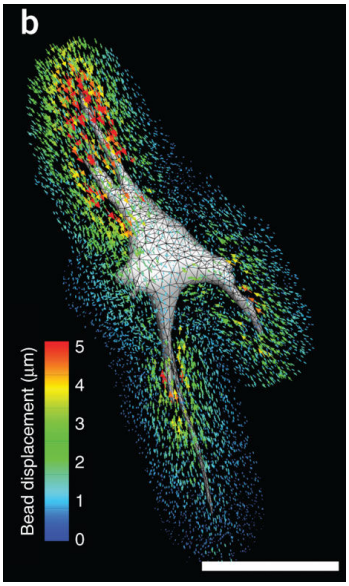

Figure 5: Figure taken from Legant et al. 2010: cell induced hydrogel formations and construction of a discretized Green's function.

Another common method to determine the forces exerted by cells is Traction Force Microscopy (TFM). TFM relies on the tracking of particles that are embedded in ECM material, whose position will be altered as cells crawl across (2D) or through (3D) the ECM. The particles will usually be fluorescent to facilitate tracking. A few years ago, this method was extended to 3D in (Legant et al. 2010)[71] allowing for tracking the forces in more biologically relevant situations. The ability to track the forces of cells migration on ECM material has been used to research topics such as how the ECM affects protease activity[31] and tension across vinculin.[72] One advantage that is immediately apparent is the fact that this method, unlike micropillars, can be extended to 3D. An additional, benefit is that, since the cells are in contact with ECM, the cell movement and behaviour should reflect in vivo cell migration more closely than the coated micropillars. A limitation of TFM is that the method is only well suited for force quantification in well-defined linear ECM materials. This means that force cannot be quantified on the natural ECM material (e.g. collagen) as this has highly non-linear force response. A recent work, however, described a method to determine the force using TFM even in non-linear biopolymer networks.[73]

While the above methods seek to elucidate the internal force composition of cells by monitoring the forces exerted on the environment, a more direct approach would be to measure these forces using internal force sensors directly. FRET-based tension sensors are an excellent tool to use for such applications, with the sensors being able to sense conformational changes in proteins and tension across molecules in the pN range. FRET tension sensors use two fluorophores in close range, separated by a flexible/stretchable linker region. The linker region allows the two fluorophores to move apart (depending on the geometry of the sensor) under force loads.

The two fluorophores have different excitation spectra, with only of the two being capable of being excited by the excitation beam of a FRET setup. The excited fluorophore is then able to excite the other through a process known as Förster Resonance Energy Transfer (FRET).[74] The degree to which the second fluorophore is excited will depend on the physical distance to the excited fluorophore, whose spatial separation requires force across the whole construct, making the emission of the non-excited fluorophore an indication of force across the FRET construct. In in vivo applications, a FRET construct is created by inserting a FRET tension sensor into a molecule of interest, allowing for evaluation of forces across it. While the generation of FRET construct can be hard, many molecules involved in cell migration have already been made by other groups[75], with sensors for molecules such as FAK[76], vinculin[77], and alpha-actinin[78][79].

1.8 Motivation

Seeing the state of the field and the current challenges, we propose the methods listed below. With this proposal, we hope to address various open questions and enable future explorations into 3D cellular migration.

The previous sections should have shown that AMT and MAT are processes that have significant medical relevance. As mentioned, various factors (both mechanical and biological in nature) have been shown to induce MAT/AMT(-like features). Since the silencing of entire pathways or a certain gene may not represent the natural way in which cells transition in vivo. Changes in the mechanical properties of the environment naturally occur when cells migrate through the body.[80] We therefore wish to study MAT/AMT of cells by mechanical constriction.

The experiments we propose in this proposal should produce an overview of the effectiveness of AMT/MAT of many different cell types. For various cancer and healthy cell lines we will explore the conditions that lead to AMT/MAT. This will allow for direct comparison between different cell lines and may reveal different approaches to MAT/AMT between (groups of) cell lines. The data generated by this paper can then be used as a reference point by other groups that wish to study AMT/MAT under non-natural circumstances, e.g. in the presence of a new medicine or after inhibition of a certain pathway.

As mentioned before, the recent interest in microtubules as more than just regulators of integrin recycling and factor transport in migration, opens up interesting new research options. Our proposed research will address the possibility of force exertion by microtubules as a potential regulator of focal adhesions and cell migration. Additionally, it could answer questions about MT stabilisation around focal adhesions.[81]

Thirdly, revealing the roles of spectraplakins and whether they actively carry a force load would reveal a lot about their function and their currently poorly studied regulatory domains. The loss of MACF1 (a spectraplakin) has been shown to cause defects in migration when depleted.[82] New findings related to the mechanical properties and possible mechanosensitive nature of spectraplakins could pave the way for research not only into spectraplakins, but also into other Microtubule-actin crosslinking proteins. We intend to achieve this by creating a FRET version of MACF, allowing for the characterisation of the forces across MACF in vivo. As spectraplakins are also large proteins, they are likely to have various undiscovered or poorly characterised protein interactions, whose future discovery could be facilitated by this FRET construct.

Lastly, this research would add a FRET construct to the large list of fret molecules related to migration that are available (see later section). This would allow future research in which more complicated questions about force distributions during migration or MAT/AMT could be addressed.

2 Platform for the study of MAT/AMT

2.1 At a glance

The core aspects of migration we wish to address with this platform are as follows:

- Is it possible to create a platform that can be used to study MAT/AMT in a high throughput fashion using mechanical constraints in realistic conditions?
- How do various cell types transition between migrational mode (velocity and morphology) in natural conditions?
- How do the intra- and extracellular forces localise for different cells types during AMT/MAT?
- How do the key regulators Rac1 and RhoA behave during MAT/AMT?

- What are the effects of different ECM materials on the transition timing and mechanism?

2.2 main

Here we will propose a design for a system that can be used as a standardised platform for the study of the Mesenchymal-to-Amoeboid Transition (MAT) and its counterpart, the Amoeboid-to-Mesenchymal Transition (AMT).

(Anguiano et al. 2017)[83] recently developed a microfluidics method to study cell migration in cancer, which they demonstrate to be able to image migrating cells with high throughput and with great control over the morphological and biochemical environment wherein the cells migrate. Often experiments are carried out in different conditions, making comparing results a challenge, and often follow up research is carried out when results conflict with each other. A prime example is the conflicting results regarding the interaction between FAK and its FERM domain, which required follow up research by (Ritt et al. 2013)[84].

This platform seeks to provide a way for researchers to reliably induce the migratory mode transitions. It would do so by modifying the physical properties of the environment, to which cells would respond to through mechanosensitive mechanisms and subsequently switch migratory mode. By not inhibiting a specific molecule to induce the transitions, one avoids the risk of also disrupting other signalling processes inside the cell or more generally causing non-biologically relevant situations. Thus, a physical mechanism as the causal agent for the transitions will allow for the study of them in a more natural environment.

The platform described here is based on various studies. Work done by (Srivastava et al. 2017)[85] allowed for cells to be confined during migration. By having a plunger system, they were able to induce a switch in type of migration from primarily pseudopodial to bleb driven in dictyostelium. While not a complete transition in the sense of AMT or MAT, it showed that pressure was enough to cause a switch in migratory type.

In (Liu et al. 2015)[55] the effects of confinement on cell migration modes in 2D/2.5D were studied. They could induce transitions in migratory modes in cells by spatially confining them. The confinement was achieved by lowering a glass plate onto the cells, thus confining them in the z direction. The height of the glass plate could be controlled, allowing for granular control of confinement. Additionally, the amount of substrate that cells could adhere to (via integrins) was also controllable, allow for even greater control. With this setup, the group was able to induce MAT.

Other work by (Pathak et al.)[86], carried out experiment in 'true' 3D, with cells being confined laterally while migrating in a 3D setup. This setup was rather low throughput, and only limited the dimension of the pores laterally. While limiting the variation in migration channels in one dimension is relevant (as in natural situations pores may often not be perfectly circular), in this setup we choose to allow the radius of the pores to be altered, essentially restricting pore size in a 2D plane rather than 1D.

A more thorough review of the current state of confined cell migration can be found in (Paul et al. 2016)[87].

In order to facilitate research, the methods used ought to be easy and fast to fabricate, and ideally possible with most tools available in most research environments.

2.3 Fabrication

We wish to create a setup that can easily be reproduced and modified by other groups wishing to study the same phenomenon.

To do this, we propose a setup that uses similar techniques and concepts as a recent platform developed to study the effects of pore size and nuclear size by (Lautscham et al. 2015)[88]. The authors created a setup that allowed for high throughput of 3D cells migration. In their setups, many cells (in parallel) migrated through a sequence of increasingly narrower pores. This setup allowed not only to study migration in a natural 3D setup, but also to study many cells at the same time.

In our setup, however, we have no need for a continuously varying pore size. At the most basic level, we require a narrowing of channels that would spatially confine cells that are migrating. These narrow sections would then run straight for a length that yields stable migration in the confined channel. and ultimately exiting in a widening of the channel again.

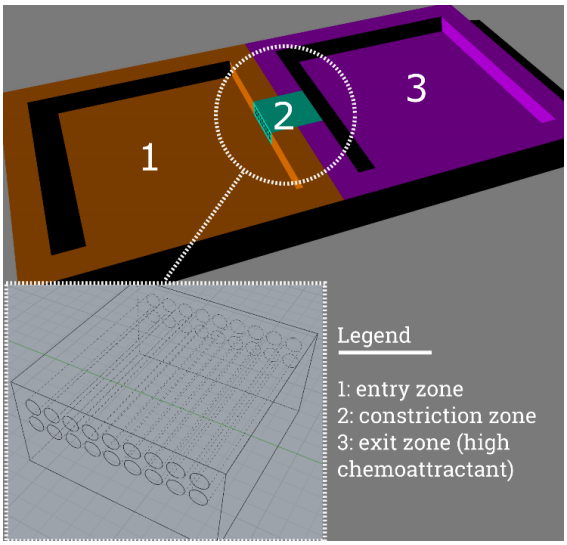

Figure 6: general design of platform, inspired by (Lautscham et al. 2015)[88]. Constriction zone depicted in its simplest form.

Some consideration will have to be given to the

exact geometry of the constriction zone. While we can make some heuristic arguments about what shapes may prove to be desirable, validation of various candidate shapes is preferred. These will be listed below in the experiments section. The most trivial and minimal shape for the narrow sections would be cylindrical channels. The round opening would match the roughly round shape of the cells, and of course be radially symmetric, introducing no biases in specific directions. Yet other shapes may also have their advantages. For example, making the narrow sections, to which we will from now on refer as the constriction zone, rectangular (thus providing a square opening), may allow for better diffusion of nutrients to other parts of the cell (other than just the front and back). This could physiologically more relevant, as in vivo one would expect nutrients to still be able to reach most parts of the cell.

Additionally, having the constriction zone be an extrusion of a 2D template, may also not prove to be desirable. With such a setup, the transition from initial zone to the constriction zone may be too sharp for cells to enter. Adding a narrowing taper to the constriction zone may make the spatial confinement less of a hard transition, facilitating cell migration into these regions. Adding such a taper may significantly improve fabrication complexity.

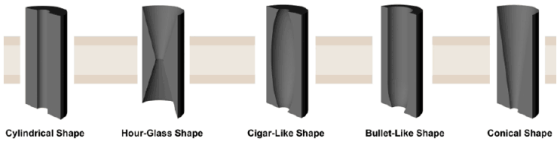

Figure 7: Image taken from(Zhang et al. 2015)[89]

The constriction zone region and platform could be generated using a microstructured template. This template could then be used as mould for PDMS, allowing the template to be used many times, and for many different labs to use the platform.. This approach would mean the platform could have broad adoption, or at the very least a low barrier for other groups to either verify experiments carried out on it or test new hypotheses with it.

The finished mould will then have two chambers separated by the constriction zone. The constriction zone is to be mounted with the passages in the horizontal plane. Cells can be seeded in either rest zone (depending on symmetry and taper symmetry). The addition of chemoattractants will then cause the cells to migrate from one chamber to the next, through the constriction zone.

The mould (including the constriction zone) can then be filled with an ECM of choice. Natural choices would be collagen, or Matrigel® (Commercial product by Corning®). ECM can be poured in the liquid phase and left to solidify in the mould.

2.4 Components

The following components are thus required for this setup:

- Incubation chamber to keep the cells and platform at constant 37 degree Celsius and constant CO2 levels.
- Master to mould PDMS platform from (with PDMS supply)
- ECM materials (e.g. collagen)
- Chemoattractant (see further sections)
- Microscope Setup (see section ‘’imaging”)

Given the components, it should be apparent that the setup can be modified with relative ease. The components are readily available in most laboratories, or available for purchase.

2.5 Quality Control

Depending on the choices of ECM materials, characterisation of the rheological properties of said ECM may be required for cross-comparison with other studies. Commercial products may have well (pre)defined properties, but materials that are non-commercial may have some variability, thus requiring measuring these properties. A commercial rheometer will enable such measurements to be done with ease. Parameters such as the storage and loss moduli can be measured.

As mentioned before, it is important that nutrients can still diffuse to all sides of the cells. It would therefore be useful to characterise diffusion in the setup for the geometries. A method to do this is described in (Anguiano et al.)[83].

It will be essential to verify that at 1) (certain lines of) cells are able migrate across the constriction zone and 2) that (certain lines of) cells do indeed undergo transitions in this region (for certain geometries of constriction zones). We are confident that such geometries exist because, as mentioned before, the group (Lautscham et al. 2015)[88] managed to induce significant morphological changes in cells in a similar setup, and the group (Liu et al. 2015)[55] was able to induce transitions by spatial constraining cells. These two references suggest that both conditions can be met. This does not, however, indicate how many cell types will exhibit both behaviours. Each geometry may be specific to each cell type (an undesirable, but possible outcome) or be applicable to various cell types. The former case will make comparing measurements harder for different cells types, but still allow for the study of the phenomenon we are interested in. It will be essential to find the geometry that induces transitions in the widest ranges of cells.

2.6 Chemoattractants

Due to the constriction region requiring internal remodelling of the cells (as the cells will have to adapt their morphology to 'squeeze through' as in (Lautscham et al. 2015)[88]), cells may not wish to migrate through this region. Additionally, having cells migrate at their own desire will limit throughput significantly. The solution to this problem is to use chemoattractants to induce cell movement through the constriction region.

2.7 Imaging

In order to do high resolution imaging of the cells migration in 3D, an imaging method capable of keeping up with the speed of cell migration. Cells do not migrate at a staggering rate, with top speeds of a few micrometres per second, which means the temporal resolution of the imaging setup needs not be of the highest order for the characterisation of general morphology of migrating cells.

For experiments that require high time resolution in order to track fast processes such as cytoskeletal dynamics, we suggest a method described in (Dean et al.)[90]. The authors describe a method to parallelise light sheet microscopy, allowing for fast 3D imaging with high resolution and minimal photobleaching. The downside of this setup would be that only a single cell can be tracked at a given time, limiting throughput.

2.8 Image Analysis

Having a 3D imaging setup means that the amount of data to process will be substantially larger than 2D setups, a problem made worse by the fact that we want to capture a process unfold over time, further increasing the dimensionality of the image analysis.

Recent work by Abbasi et al.[91] has demonstrated an effective toolbox for the analysis of cell migration images. They analysed a cell line of mesenchymal cells and discovered to distinct subpopulations of mesenchymal cells. While their analysis focussed on 2D cell migration, their results demonstrate the ability of their toolkit.

These examples show that there is already extensive literature available for the tracking and analysis of cell migration. These methods will have to be extended to suit the particular questions that are asked in the proposed experiments section, but can be built on the current methods in the literature, requiring no novel features.

2.9 Parameters

This setup could be modified in many ways. The geometry and taper of the constriction zone can be modified, the density and crosslinking of the matrix in the PDMS mould can be modified. Different cell lines can be selected. Levels of chemoattractant can be controlled.

2.10 Cell types/lines

For this setup we wish to try a large range of cell types. The Cells we suggest are based of the cells used in (Liu et al. 2015)[55]. This means we wish to use: NHDF (normal healthy Dermal Fibroblasts, RPE1(retinal pigmented epithelial cells), NHEK (normal human epidermal keratinocytes), MCF10a (human breast epithelial cells), HeLa F (fast moving HeLa subclone), MEF (mouse embryonic fibroblasts), MDA-MB-231 (human breast adenocarcinoma), HT29 (human colon adenocarcinoma), A375M2 (shown to switch between amoeboid and mesenchymal mode[92] and are amoeboid on soft collagen[93]), HT1080 (human fibroscarcoma), MDCK (Madin Darby Canine Kidney, shown to have 2 migration modes), C2C12 (mouse myoblast), HEK293 (human embryonic kidney, has been shown to be able to move amoeboidly[94]), RPE1 (can change in migrational mode), MCF10A (can undergo (partial) MAT[95]), Jurkat (immortalised T-cells), walker 256 carcinosarcoma, WM266.4 cells (metastatic melanoma line, exist in 50:50 mesenchymal amoeboid ratio, switch often[96]). The last cell line may prove to be too dynamic for this experiment, but is also the one that would be most ''eager" to switch migrational mode. Cells omitted from the aforementioned work are: A735P, HeLa VL, HeLa MyH9, HL60 (promyelocytic leukaemia), Human Monocytes.

2.11 Experiments

in order to find a good geometry for the platform, we will use 3 different ECM: 1) Collagen 2) Matrigel®, 3) Collagen-Matrigel mixture. Having three different ECM materials will allow for exploration of different cell adhesion during migration. We will then test various geometries of the constriction region opening. The shapes we wish to test are given below. Motivations for each in order: radial symmetry, diffusive space (minor surface area increase), diffusive space (large surface area increase), diffusive space (large surface area increase; narrow). Each shape will have the same minimal inner dimension. Performance of each shape will be evaluated by the number of cells migrating through the passage over a large time span averaged over a large number of cells. We will assume that the highest rate will be the best shape for our assay. As the circular shape is the simplest to manufacture, testing will begin with this shape. If issues with cells migration are found that may be attributable to poor diffusion

of nutrients, the other geometries will be tested.

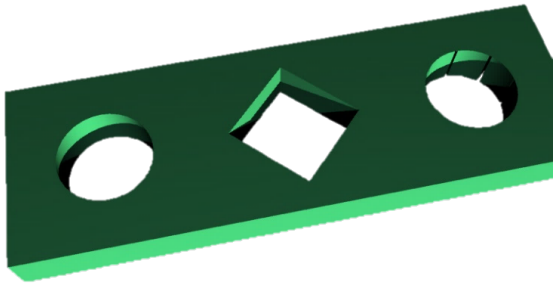

Figure 8: Constriction zone profiles: round, square, round with diffusive slits.

The selected shape can then be tested with different tapers, to assess if adding tapers to the shapes adds any increase in migration, and whether this increase is worth the trade-off in manufacturing difficulty. This should then yield a platform that we use to answer questions and characterise the processes of transitions of migratory mode – which is ultimately what we are interested in.

2.12 transition times-based characterisation

This setup would allow for repeated induction of transitions in cells. This means one can monitor many cell transitions with relative ease, allowing for robust statistical analysis. One of the direct applications and questions that has (to our knowledge) as of yet not directly been addressed, is on the duration of transitions in a biologically relevant situation.

2.13 Probing Collective cell properties

In a series of experiments, the transition times, both for MAT and AMT would be monitored for large number of cells. The above will be carried out for various cells types, allowing for the probing of differences in ''eagerness" to transitions for different cells types.

Optionally, the above will be carried out on various ECM stiffness levels. This could reveal whether each cell has an optimal transition setup. Additionally, it could reveal how sensitive each cell type is changes in cell stiffness compared to transition speed, allowing direct comparison to previously recorded migratory speeds.

Likewise, varying other physical properties of the platform and monitoring effects on transition times could reveal information about the specific cell types' response to environmental cues.

Besides revealing information about the dynamic response to the environment and characterising an important parameter of migratory transitions (transition times), such characterisation would also be highly relevant for cancer research. As metastasis in cancer requires switching of migratory modes in order for cancer cells to migrate to other parts of the body[56] (see previous sections), it is important to know what conditions facilitate this. While the magnitude of transition times themselves may not be as useful, the optimal conditions (i.e. the change in transitions time with varying parameters) would be. This would rely on the assumption that the transition time accurately reflects the ''eagerness" of cells to transition modes. We believe this is a reasonable assumption.

Repeating the previous set of experiments for various cancer types and ECM compositions could reveal information about the effect of 3D environment on metastasis for a wide range of parameters.

2.14 Individual cell behaviour

While the previous proposed experiment would reflect collective behaviour averaged over a significant number of cells, the measurements are ultimately carried out over individual cells. This allows us to also probe individual cell dynamics, probing the heterogeneity within the same cell type. We therefore propose the following set of experiments to characterise this.

By tracking individual cells, we can have the same cell undergo multiple transitions (of the same type). We can do this by placing a second construction region after the first. This allows us to eliminate cell to cell variation and probe how much variation is present for transition times for a cell. Since we use the same cell (thus with the same resources etc.), and keep the environment (the setup and ECM) roughly the same, any changes in transition times could either be attributed to stochastic processes in the cell, or as a result of the previous transitions.

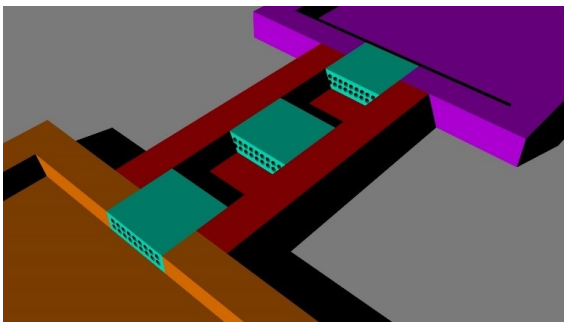

Figure 9: Repeated constriction zones to study reversibility of transitions.

2.15 Morphology-based characterisation

Since the transition time only reflects part of the transition story, it would be useful to also evaluate the morphological changes during transitions. By analysing morphology of cells during transitions, other research has been able[91][55] to determine novel transition pathways and subtypes of migratory modes. Such behaviour would be lost in transition time char-

acterisation. In the following set of experiments, we extend the characterisation to taking cell morphology into account.

The first challenge would be to develop an assay that can classify shapes into various groups. This is different from the initial classifier, that merely determined whether cell was migration amoeboidly or mesenchymally.

We suggest using a system that takes information of both the physical morphology of the cell and certain molecular markers into account. The shape of the cell could be classified into subgroups, as mentioned before. The molecular markers and their localisation can then also be used to distinguish subtypes, as a complementary input for a classifier. This way types of migration modes can be distinguished both on the external organisation (morphology) of the cell and the internal organisation (molecular markers). We suggest integrins (and they activation), Rac and Rho as potential molecular makers.

2.16 ECM transitions

By creating a setup wherein the ECM stiffness differs on either side of the transition region, (where one side is enough to induce a transition in the transition region), and by varying stiffness on the side where the cells migrate to, we can mimic cells transitioning to different environment through some region of constraint (transition region). This presents a manufacturing challenge, as it would be critical that the two different ECM materials in either chamber flanking the constriction region meet in the construction zone. A possible solution could be first pouring the first ECM material in the setup that is positioned with the constriction holes aligned with gravity. This would allow the still liquid ECM to be poured to about halfway the constriction zone, and then left to polymerise. After this the secondary ECM material could be added, with minimal mixing of ECM material. Particular care will have to be taken to avoid wetting problems where the two ECM materials meet. Such an asymmetry in ECM flanking the transition region, we speculate, can lead to two potential behaviours upon undergoing AMT.

1. We observe some form of hysteresis, where a certain stiffness threshold on the exit ECM must be reached before we observe AMT
2. We do not observe hysteresis, but observe a difference (lengthening) of the transition time of AMT with decreasing stiffness.

If the first is observed, this may imply that a transition to a different mode introduces some (partially) irreversible change in the internal organisation of the cell, or an asymmetry in the transition process. The second option may also manifest itself as a hysteresis effect, if the transition times are exceedingly long. In this case only a significant increase in stiffness will introduce a transition; falsely suggesting the presence of hysteresis.

2.17 Traction force microscopy

While the morphology of cells can tell us about the internal changes in the cells during transitions, the cell transition is ultimately a shift in the way cells exert forces on the environment.[97] It would therefore be of tremendous interest to probe how the forces on the ECM changes as the cells undergo AMT/MAT. To speculate, we expect FAs to be degraded during MAT, which would decrease forces at those sites locally, as well as possibly increasing forces on the remaining FAs. Additionally, we wonder if we see a local response (FAs only being degraded at the site entering the constriction zone), or a more global response (FAs at the rear of the cells also being degrades at an increased rate).

In order to probe forces in 2D cell migration, TFM (described earlier) can be used. A group[73] developed a method to extend TFM to 3D measurements. TFM measurements are restricted to mechanically more well defined materials, for example polyethylene glycol (PEG).

A recent method , however, developed a method to infer forces by tracking beads even in natural (nonlinear) ECM. They achieved this by incorporating a continuum description into existing mechanical models. While this meant resolution was restricted to the region where this continuum description holds, it still mean they could achieve an acceptable resolution (see reference). This would allow for the study of forces in physiological ECM materials, albeit at a decreased resolution.

We propose running the previous experiments in Collagen, Matrigel®, Collagen-Matrigel, and PEG. The first three could use the continuum model for force quantification, while the last option could make use of the model assuming linearity of the ECM. The cells chosen would be the selection of cells that will have been shown to undergo AMT/MAT in the platform. Of particular interest will be the MDA-MB-231 cells, as they were used in the continuum, model and can therefore be used to verify and extend that paper's results. One challenge in this case would be the fact that the dynamics of the ECM material may be different around the constriction zone. This because the ECM material is now locally constrained. Careful force measurements will have to be conducted to see whether measured forces are representative in the constriction zone. If they are, we will have to limit force measurement to the entry and exit zones, in which there are no such problems. This will allow us to determine whether cells return to the same force exertion levels after transitioning through the constriction zone.

2.18 Rac/Rho switching during transitions

As mentioned before, the antagonistic nature of RhoA and RAC1 are important determinants of amoeboid and mesenchymal migration respectively. This means that during transitions, their relative expression and activation should be altered considerably. By being able to study Rac/Rho activation in a true 3D transition setup will allow us to answer questions about how these factors interact and regulate migration in a biologically relevant manner.

To monitor this, we can use FRET constructs. The base for the FRET sensors we deem suitable for this setup was developed in (Itoh et al. 2002)[98]. This group developed a FRET probe which they named Raichu (Ras and interacting chimeric unit). This is a fret construct that can be used and modified to work with many GTPases. The authors developed a Rac FRET sensor in the same year, and in 2003, a Raichu-RhoA FRET construct was generated.[99]

We expect cells moving in amoeboid fashion to have higher expression levels of RhoA (and ROCK) and Rac is active at the leading edge in mesenchymal migration.[100][27]

Validating the current opinion on Rac/RhoA dynamics in cell migration (AMT/MAT) and characterising how these expression levels and activation are regulated during transitions could help further map these key regulatory factors. Particularly interesting will be to probe whether these two components get (in)activated/deregulated at the same time, or whether one lags behind the other. Additionally, it would be important to probe whether the changes in expression occur co-locally, or in different spaces, which could suggest a linked mechanism.

3 Spectraplakins

3.1 At a glance

The core aspects of spectraplakins we wish to address with the aid of a FRET Construct are as follows:

- Is it possible to generate a MACF FRET construct that still retains all the wild type functions and doesn't alter the proteins properties significantly?
- Where does MACF localize and how is this different in amoeboid and mesenchymal migration?
- How are the forces distributed in this migrational mode and how does this relate to other forces in the cell?
- How will disrupting the regulatory regions of MACF affect the forces across it and cell migration in general? Can we rescue this behavior?
- What roles does ELMO play in relation of MACF, and how are forces across MACF related to ELMO? Can ELMO alone enough to anchor MT?
- How will the forces across MACF and MACF-deficient change during AMT/MAT? How will MT (de)stabilizing agents modify transition rates (as compared to part I) and can we (partially) rescue the effects of MACF-deficient?

3.2 main

It is increasingly becoming clear that crosstalk between microtubule and actin cytoskeleton plays important roles in cell migration.[33] One type of protein that can enable crosstalk between these highly dynamic cytoskeletons are spectraplakins. Spectraplakins are large (¿500kD) master orchestrators] proteins that can physically link the actin network to microtubules. They concentrate at growing microtubule ends through association with end-binding proteins on MT. This means that they belong to the class of plus-end tracking MT molecules.[101]

3.3 Structure

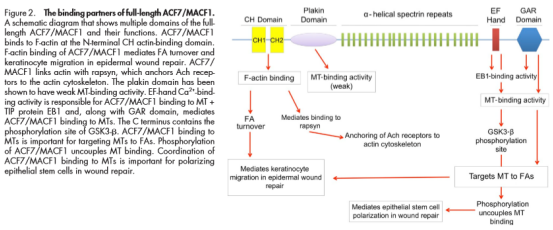

Figure 10: Taken from Suozzi et al. master orchestrators, N terminal domain on the left of the image.

As we can see from figure 10, the structure of MACF1 (also known as ACF7) can be divided up into 5 sections. On the N terminal side, we find two Calponin Homology (CH) regions, termed CH1 and CH2. These regions bind to actin, enabling the interaction with the dynamic actin cytoskeleton during migration. Towards the C terminal domain, we find the plakin domain. The plakin domain is believed to have been evolved from spectrin repeats.[102] This plakin domain has been shown to bind to various molecules such as integrin, BPAG2 and Erbin[102], as well as containing a SH3 protein-protein binding domain.[103] Next to this plakin domain we find a region of spectrin repeats. These regions act like a spacer and give flexibility to the protein.[104] A recent group has created a version of MACF that lacks these spectrin repeat and plakin regions, which they called TipAct. This modified version still contained the MT and actin binding domain. Further towards the C terminal domain we find the EF hand region. This contains two EF-hand calcium binding motifs that do not affect MT binding.[105] At the C terminal domain we find the GAR

domain of spectraplakins. This section binds to microtubules, and stabilises them.[106]

Since we have proposed a novel system to study the transitions between the migratory states of most animal cells (Part 1), we here propose experiments to study the highly dynamic cytoskeletal crosstalk both inside and outside these transition events.

The reason why we think studying this crosstalk in the broader context of AMT/MAT is a promising avenue to explore is the fact that the cytoskeletal makeup is structurally different in amoeboid and mesenchymal migration (see earlier sections). This suggest internal reorganisation of the cytoskeleton takes place during these transitions. If there is significant crosstalk between networks, it is well possible that these networks influence each other during transitions. It would well worth it to characterise the spatiotemporal regulation of these networks and their interactions during AMT/MAT for this reason.

Central to answering these questions would be a new FRET construct. This would be a FRET construct of the MACF1 spectraplakin. We believe that spectraplakins could be a promising FRET construct, as both the actin network is under stress during migration and MT are known to be able to generate forces, lending credibility to the idea that spectraplakins also experience forces during cell migration.

The question then remains whether this force would have any biological significance. Spectraplakins have been shown to have other active regions[104], beside the actin and microtubule binding regions, suggesting more than a simple crosslinking function. The presence of many mechanosensitive molecules in the cell migration machinery one may be led to the possibility that spectraplakins may themselves also be mechanosensitive. This would mean that they could exert some regulatory function depending on the force exerted on the molecule.

A FRET construct could, provided generation is manageable with the current techniques, characterise the forces on spectraplakins over time and space. This, paired with our AMT platform could allow us to reveal crosstalk of the cytoskeleton in a new way. FRET, being fundamentally a fluorescence based technique, it can be conducted on live cells, facilitating long-term (minutes/hours)[107] study of the same migration cells, potentially allowing to discriminate differences between individual cells from noise.

Interestingly, in 2014[108] a human disease caused by alterations in MACF1 (ACF7) was reported. The disease was caused by a duplication of a large part of the gene coding for MACF1. This reduced expression levels of all isoforms of MACF1. This highlights that MACF1 and spectraplakins are important for human health.

3.4 FRET construct fabrication

As with any FRET sensor, a tension sensing module (TSMod) will have to be selected and inserted in the right domain in order for FRET to function. This TSMod (in most conventional applications) consist of 2 Fluorescent molecules (generally proteins if one wishes to genetically encode the FRET sensor) that must be inserted in a domain in a protein that bears forces or undergoes conformational changes. It should be notes that current sensor designs convey information about the magnitude of the force, but not about the direction, which in case of cell migration (in which the cytoskeleton has a distinct spatial organisation) would be highly relevant information. Yet, even with the inability to track the direction of the forces, the magnitude of the forces could provide meaningful information about the temporal dynamics of a system.

Finding the right TSMod for this particular problem is not trivial, as it is currently unknown in what force range (if at all) spectraplakins operate. A sensor that is very accurate by today's standards is described in (Grashoff et al. 2010)[109] and has a force sensitivity of 1-6 pN. This TSMod has already been used in Vinculin. More importantly for this problem, a group[110] has recently been able to use this TSMod in spectrin, which is present in Spectraplakin molecules, including AFC7. They inserted the TSMod sensor in between the 8th and 9th spectrin repeat. This approach seems like it could be as successful for spectraplakins. The benefit of using an existing tension sensor is that one does need to carry out extensive calibration, reducing time needed to carry out the research.

Great care must be taken where the TSMod will be inserted, as to not disrupt protein function and to capture the force bearing of the molecule. While the general structures of spectraplakins is known, extensive characterisation of every part of the structure is not available. The crystal structure of the F-actin binding domain (CH domains) of dystrophin and utrophin has been characterised, similar to those of the spectraplakin actin binding domain.[111] The crystal structure of the plakin domain is also known.[112] Additionally, when placing the TSMod, it must be inserted in regions that do not have a functional site (e.g. a protein binding / interaction site). In the case of spectraplakins, this poses a challenge, as it is known the molecules have protein binding sites, of which many may be as yet undiscovered. It will therefore be critical to try multiple sites, and carefully monitor effects on living cells to ensure the natural functioning of the molecule is not impaired.

Once the possible insertion sites have been selected, generation of the protein construct in vitro can take place using either 1) restriction enzymes 2) overlap extension 3) Gibson Assembly. For this research, Gibson Assembly seems most fitting, as assembly kits are commercially available.

Thus, after selection of sites and generation of DNA, the protein constructs with TSMod inserting can be validated for basic functionality. Grasshoff et al, 2010 recommend the following set of chemical validation experiments before using the construct in vivo.

1. Measure the molecular weight of the protein construct (using Western blot and gel electrophoresis) in order to validate that the protein is not subject to degradation

2. Conduct FRAP experiments (with both the wild type construct tagged with the acceptor fluophore and the full construct to see if the diffusion coefficient and localisation properties are not markedly altered.

3. Conduct Co-immunoprecipitation assays to validate whether all known binding interactions are maintained in the construct.

4. Validate whether the construct can rescue phenotypes induced by knockout of the endogenous version of the protein.

3.5 Foreseen challenges in generation

As mentioned before, it is critical to ensure that all functional regions of the FRET construct are unaltered in the process.

MACF1 comes in various isoforms, with each isoform being expressed to different extent in different tissue type.[104] Some of these isoforms differ quite substantially from the type depicted in figure 4, which could complicate measurement. One isoform does not contain and ABD, which will in all likeliness not locate to the region that the ones with ABD (which we wish to study) do. This could cause erroneous FRET signals.

The group that characterised a spectrin FRET construct inserted TSMod in some of the spectrin repeats, which seems like a tempting region to attempt for spectraplakins. One potential danger in this region would be the ATPase region first discovered in (Wu et al. 2008)[113]. The authors propose the ATPase region lies in the spectrin repeat region. Careful evaluation of this activity will have to be conducted after construct generation. This is supported by the fact that the deletion of a few spectrin repeats has been observed to cause mild dystrophy[114], suggesting that there is some functional regions present in the spectrin repeat region of spectraplakins. The last spectrin repeat has also been found to mediate binding with protein called ELMO.[115]

The other region that is not critical for Actin or Microtubule binding is the plakin region, making this another potential target for FRET module insertion. Due to the various active regions discovered to date, however, it may prove to be very hard to find a location that does not interfere with any of the active regions in that section of spectraplakins.

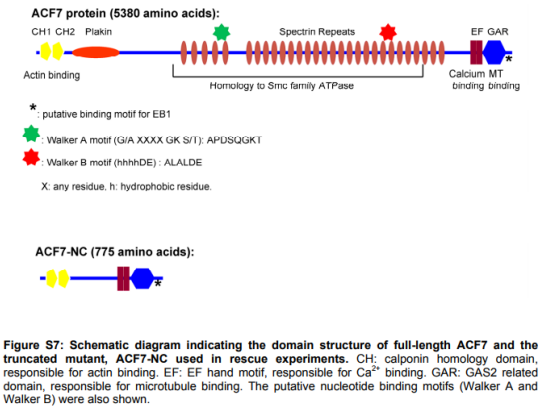

Figure S7: Schematic diagram indicating the domain structure of full-length ACF7 and the truncated mutant, ACF7-NC used in rescue experiments. CH: calponin homology domain, responsible for actin binding. EF: EF hand motif, responsible for Ca^{2+} binding. GAR: GAS2 related domain, responsible for microtubule binding. The putative nucleotide binding motifs (Walker A and Walker B) were also shown.

Figure 11: Image taken from supplementary material of (Wu et al.)

3.6 Experiments

Provided the creation and expression of a FRET construct of MACF1 is successful, we can use this new construct to test hypotheses that were previously difficult to test. These questions revolve around how the crosstalk between the actin and microtubule cytoskeleton takes place, and about the exact roles of spectraplakins in this process.

To validate that the localisation properties of MACF-FRET are unchanged, we propose a setup wherein we express MACF-FRET in vivo combined with mCherry-EB3 (End Binding protein family member 3). EB3 will bind to the +end of microtubules, allow us to assess whether MACF-FRET localises to areas consistent with previous findings. Previous experiments have shown that MACF is a +TIP factor. Work by (Applewhite et al. 2010)[116] found that SHOT, a drosophila homolog MACF, was found to trail behind the ends of MT. Interestingly, another group[117] found that their modified version of MACF did localise to the end.

3.7 Spatial Distribution

Unlike simple fluorescent constructs, FRET-MACF allows to not only visualise the distribution of MACF throughout migrating cells, but also the distribution of force loads on the molecules.

Research by (Balzer et al. 2012)[118] has found that upon spatial confinement, cell migration depends largely on microtubule dynamics, whereas disruption of F-Actin had little effect. This leads us to suggest that in this case, there should be limited crosstalk between the different filaments, and thus we would expect to see low FRET. Studying whether MACF still localised to force exerting MT in this context would also work towards answering the bigger question: does MACF (or spectraplakins) play a role both in amoe-

boid and mesenchymal migration, or just in mesenchymal migration?

Since it has been observed that amoeboid migration relies on cortical contractility and hydrodynamic flows of the cytosol, rather than on adhesion based force exerting, we have a very different actin structure in said migratory mode. In this context, we can determine (1) whether spectraplakins still localise with MT plus ends during amoeboid migration and (2) whether any force is transduced through spectraplakins in this migratory mode. Since Amoeboid migration required contraction at the rear of the cells, in contrast to in the front of the cells, we expect to see this reflected in FRET levels, if microtubule depolymerisation[119] contributes to this process. This allows us to answer not only the question above, but also reveal whether spectraplakins interact with different type of actin structures in different migration modes and to what degree.

To address the first question, a setup where cells will be monitored in different migratory modes, analysing the localisation and intensity of the fluorescence signal emitted by FRET-MACF will be used.

For this experiment we will monitor fluorescence in 3D ECM media. Three cells lines will be used: NHDF (mesenchymal), A375M2 (hybrid), HEK293 (amoeboid; in right conditions) The first two exhibit one migratory mode only, and thus stably reflect the mechanisms at play for said migration mode. The third cell type exists in both migratory modes, which allows us to eliminate any differences between cell types and see if the general observations hold.

From the intensity imaging of cells, we can construct the distinct distribution pattern of FRET-MACF in cells and therefore the force distribution in cells. Such distributions are expected to highlight a difference in the interaction of the different cytoskeleton parts (actin, MT) in amoeboid vs mesenchymal migration. For mesenchymal migration, we expect to see an increase in intensity at the cell front (under the assumption that there is some force crosstalk between the force-exerting cell front (actin) and the MT cytoskeleton). Speculating on the distribution in amoeboid migration is more challenging, as the role of MT in amoeboid migration are less clear, with findings suggesting MT play only a limited role, and more recent findings suggesting MT are relevant33. Since force migration happens by contraction in the cell rear, and MT are able to link to the cortical (actin) cytoskeleton and are capable of exerting pulling forces by depolymerisation and this has already been observed to affect cell migration[120], we may expect to see increased localisation at the cell rear.

Of particular interest will be to what extent the signals during mesenchymal migration and amoeboid migration overlap in location and intensity.

3.8 Forces

The next question that one would logically be interested is in, is how the forces exerted by the cell relate to the forces across the FRET constructs. The forces experienced by different cell components during migration could be compared to the force experienced by FRET-MACF.

Measuring the force exerted on the surrounding (3D) ECM, using TFM[73] will allow the force exerted by cells to be related to the tension across the (MACF) FRET construct. Since we expect some variation between the force exerted within populations of the same migration type, and cells change strength of adhesion and force exertion locally over time, it would be possible to probe how an increase in force exerting is matched by an increase in FRET or whether the two variables are related in a different fashion, and how these relate in the two migration modes. Alternatively, rather than inferring forces by external beads, one could consider using an approach similar to (Grashoff et al. 2010)[72] using a separate FRET probe to determine forces across FAs. The latter would require a more complex optical setup and choice of fluorophores.

While the forces exerted by a cell on the ECM is a highly relevant metric, the relative force distribution in different parts of the cell, which is highly inhomogenous[72], may be as important. By comparing the forces across MACF and other key force bearing migration-related proteins, we can more precisely map the internal regulation of the cell's internal force balance during migration.

There are two key proteins that are known to play key force transducing roles and interact with the actin cytoskeleton during mesenchymal migration. These proteins are vinculin and alpha-actinin.

In order to explore the relation between forces on vinculin and FRET-MACF, we intend to create mesenchymal cells from NHDF cells that express both FRET-MACF and the previously developed FRET version of vinculin.[77] Since we have two different FRET Constructs, it will be important to have an optical setup that can differentiate between the two signals, with minimal spectral overlap between the FRET emission spectra. As we previously proposed TSMod as the tension sensor component for MACF-FRET, a different variant of either MACF-FRET or Vinculin-FRET would have to be constructed. This is because both would use the same tension sensor otherwise, making differentiation of signals impossible. Since vinculin an important focal adhesion protein, and has been used to quantify force across FAs, revealing how forces across it relate to forces across MACF could reveal the relation between FA forces (thus cell traction forces) and the microtubule cytoskeleton.

The second protein, alpha-actinin, plays a direct role in crosslinking different actin fibres. Since MACF has the ability to crosslink MT and Actin, and Alpha-

Actinin crosslinks actin with itself, we expect both to be affected by the dynamics and organisation of actin. It would be interesting to see whether increases in tension across either results in an increase in the other, and whether this depends on the (local) actin structure. Again, probing whether the behaviour (change in force in both tension across MACF and Alpha-Actinin) is identical for cortical actin (amoeboid migration) and regular/stress fibre actin (mesenchymal).

The main challenge in the above two experiments will be the separation of emission spectra. This may require modified version of the FRET sensors, which will require calibration of each sensor again. The above experiments are intended to be conducted on mesenchymal cells, as vinculin does not play a significant role in the largely FA-independent amoeboid migration.

Having established the relation between forces in various cellular components, it will still be important to establish which component directly exerts forces of MACF. With the current knowledge of MACF, we can consider two components that could both generate or/and transduce forces to MACF: actin and microtubules. Actin is known to generate the pushing force required for mesenchymal migration, while MT have not been observed to directly exert forces in the context of cell migration (in the sense that actin does).

To modulate the forces generated by each component we suggest testing the distribution and magnitudes of forces across MACF for different ranges of (potential) microtubule forces; achieved by adding depolymerising and stabilising factors. To modulate actin generated forces, the activity of myosin could be (partially) inhibited. To achieve this, we would pick the same mesenchymal, amoeboid and hybrid cell line as before and study the cells migrating in 3D. It will be essential that this change in FRET is measured shortly after modulation of a component, in order to measure the change caused by the modulation. This way we can compare differences between forces for each cell with itself, and compare locational dependence of force changes in cells. As MACF will crosslink MT and actin, the forces across it can serve as an indicator for forces on MT and actin. Any tension on MACF should mean there is also tension on the MT it is attached to. Thus, MACF-FRET could serve as an indirect course indication of forces (tension and compression) across microtubules, which could be used to test the feasibility of the tensegrity model in vivo.

3.9 Regulation

An interesting avenue to explore with MACF-FRET would be the effect of forces on the regulatory domains of MACF. As postulated before, MACF may be mechanosensitive, with potential regulatory domains that can be accessed if the molecule is stretched under force. Such domain can uniquely be studied by this FRET construct. Thus, it is important to identify these domains and the associated proteins. While we cannot probe the individual domains, partly because some may not be discovered, and partly because those that are known are not fully characterised, we can probe the general effect of different forces across MACF. These effects can then potentially be related to the regulatory regions of MACF.

3.10 Disruption

In order to determine whether MACF has the ability to regulate the cytoskeletal dynamics in a force dependent manner, we propose to also create a version of TipAct[117] that replaces it's GFP with a FRET tension sensor. Since this molecule has none of the regulatory domains (plakin and spectrin repeats), but retains its Actin and MT binding domain, this molecule should also be able to probe the forces acting between the MT and the actin in cells, but unable to act in a regulatory manner.

By generating cells lines that express FRET-MACF and a variant deficient for the regulatory domains, we can study the effects of the regulatory domains (or lack thereof) on the forces across MACF. If the regulatory domains do not affect the polymerisation rate of MTs or the actin dynamics, we expect the forces across MACF in the deficient variant to remain the same. With this, we can determine if the regulatory region is essential for maintaining the tension across MACF.

Since we believe some of the regulatory regions may be important for cell migration, we expect to see a change in forces across MACF. We propose to try to rescue the defective phenotype (changed tension across MACF) by adding microtubule stabilising or destabilising agents. If the regulatory domains are essential for maintaining natural MT dynamics, then we should be able to rescue the cells by either adding or destabilising MTs. It is likely, however, that the regulatory domains regulate other processes that may not affect the forces across MACF itself. This could establish whether the forces across MACF are (by themselves) enough to enable migration or whether the regulatory domains are essential. Thus, if we wish to first establish whether the effect of a MACF mutant is caused through altered cytoskeleton dynamics. If we determine it is not, the effect on cells must be through other pathways, whose effect we can then relate to tension across the construct. This can then be compared to the effects of force across MACF on cells, to possibly elucidate the mechanism of the regulatory region. Of particular interest, will be studying FA maturation with the mutant MACF. FAs are known to mature (and grow in size) as force is applied across them.

3.11 ELMO

A recent study by (Margaron et al. 2012)[115] found that ELMO (engulfment and motility), a regulator of Rac signaling , interacts with MACF1. ELMO forms a complex with DOCK180, and is (in active conformation) recruited -to the membrane. The ELMO-DOCK180 complex is known to regulate Rac, a central Rho GTPase in cell migration [121][122]. Microtubules are captured at the membrane by the ELMO complex. It therefore not only directly with ALCF7, which plays a role in cell migration, but also regulates Rac activity, which is central to migration mode. Because of this, we intend to investigate this closer using FRET-MACF.

Other recent studies have identified that ELMO-DOCK180 not only plays a role in mesenchymal migration (through integrins), but also regulates cell to cell adhesions (through cadherins), suggesting an important role for this complex in cell adhesion[123]; a novel interaction with Cdc27.[124] We propose the following set of experiments to more closely study the interaction between spectraplakins and ELMO.

We propose a setup using MDA-MB231 human carcinoma cells with FRET-MACF and fluorescently labelled ELMO. The cell type was used by the aforementioned authors[115], who argue this cell type is a good candidate for ELMO research. In this setup, we could not only see the colocalization of the two proteins (as was done before), but also probe the force across MACF, which may reflect the binding state of MACF. The authors determined the region of MACF that facilitates ELMO binding was around the 17th spectrin repeat of MACF. The FRET intensity could reveal whether ELMO binding to MACF only occurs at a specific Force across MACF (which could, for example, make the binding site (in)accessible)

Since MACF can bind to ELMO, which is recruited to the membrane, it would seem possible that the physical connection of MT to the membrane is only through the ELMO interaction, rather than through the ABD. To verify this, a version of FRET-MACF could be created that lacks the ABD. The presence of the cortical actin cytoskeleton would suggest that MACF would have actin to bind to if recruited to the membrane by ELMO. If we observe significant changes either in FRET or cellular behavior, we can conclude that MACF still binds to both actin and MT, and that ELMO simply aids in the recruitment to the membrane. If the ABD is dispensable, it suggests the forces across MACF are created either by compressive forces generated by the membrane or by depolymerisation of the MT. Treating cells with either MACF or MACF-nABD with depolymerizing and stabilizing agents will provide additional evidence for either outcome, and if the latter is the case (MACF does bind to actin at the membrane in wt), preventing pulling forces by MT may allow for rescue of the MACF-nABD phenotype.

Inspired by the aforementioned work that showed a role for ELMO in Mesenchymal transition (not to be confused with AMT), we propose the following experiments to expand on this possible role. The previous work assessed MT by measuring various mesenchymal markers. The cells in question are glioma, and thus may only reflect the specifics of said tumour cell type. ELMO is also known to be able to regulate Rac Signaling.[125]

We intend to first assess the migratory speed and directionality in 3D migration of (mesenchymal) glioma. We will assay these parameters for cells with normal WT Elmo expression and silenced Elmo. (Zhang et al.)[126] mentioned the effect of silencing Elmo could partially be rescued by IL-8. This type of experiment should relatively little time due to the fact that it would use components used throughout the other experiments and would serve to verify the authors' claims with a different type of assay. The same authors found that IL-8 interacts with ELMO (through CXCR1) and that through ELMO it can induce cytoskeletal rearrangement (through Rac). We propose to probe the effect of IL-8 in WT-MACF and TipAct, which lacks the ELMO binding domain. This allows us to distinguish whether the MACF binding of ELMO is required for the Rac regulation, or whether the lack of MACF changes the positioning of Rac expression. Rac activation can be tested through available FRET sensors.

3.12 AMT/MAT

In a final set of experiments, we propose combining FRET-MACF and the MAT/AMT platform. As the cytoskeleton undergoes significant rearrangement during transitions (as shown by the difference in cytoskeletal arrangement in either migratory mode), we would expect this to be reflected in the interaction between the actin and microtubule cytoskeleton. MACF-FRET will allow the spatiotemporal regulation of the forces between these networks to be visualised. This would not capture other, non-MAFC based force transduction mechanisms between these cytoskeletal components, thus only providing a limited, but valuable, insight into cytoskeletal interactions during MAT/AMT.

For the first set of experiments, we intend to map the spatial distribution of forces across MACF during the transitions for the cells that have shown to be able to undergo migration in our platform in earlier experiments. This will reveal the natural evolution of MACF dynamics in wildtype cell migration.

We propose mapping the 3D organisation of FRET-MACF intensities of multiple cell types during MAT/AMT. Cells will migrate and undergo transitions in the platform as described earlier in this proposal. As verification that the transitions do not introduce a bias or unnatural behaviour in the cells, we will compare the organisation and tension across MACF in the cells when they are stably moving in either type

to the non-transition experiments. Spatial distribution of forces should be similar to that observed in earlier experiments.

To extend on the experiments regarding MACF-deficient, we propose repeating the above experiments on cells expressing the Regulatory domain-deficient MACF constructs.

We propose measuring and comparing the 3D organisation of forces of MACF-deficient during migration and the transition success rate of said cells. This will allow us to answer whether a lack of MACF regulatory function is needed for cell transitions, and whether this is essential or dispensable. We can compare what the change in transition times are for each cell type and compare this to the WT.

In order to see if we can restore the forces across MACF by adding MT (de)stabilising agents (similar to previous experiments, but now in context of MAT/AMT), we propose probing the effects of MT modulation on the MACF-deficient construct during transitions. This will be harder to study, as modulating MT dynamics may restore forces across MACF in mesenchymal migration but impede it in amoeboid. As a control, we would repeat the transition experiment with WT MACF in presence of these factors. With this, we could compare if we can (1) (partially) rescue MACF-deficient impaired transition (2) disrupt the natural transition efficiency of the cell types. The former will provide additional information regarding whether MACFs regulation of migration is through force across itself, or through its regulatory domains which would function through other pathways. Effects through the mutant MACF can only be caused through the force of MACF or MT themselves, while the WT may have additional effects due to its regulatory domains. Comparing these two will therefore be very informative. Both options would provide evidence that targeting the MT in cancer cells could be a feasible strategy to prevent metastasis.

4 Outlook

MACF has various isoforms, one of which (MACF1-4) has eight plectin repeats instead of the ABD.[104] Other studies into plakins[102] have found that plectin repeats allow for binding Intermediate Filaments (IF), which are other cytoskeletal filaments. This opens up the possibility that this one isoform may be able to bind IF, allowing for the crosslinking of the MT network with IFs. This would be an interesting option to explore in light of the tensegrity model[40] and since FRET versions of regular MACF will already be available, fabrication should be relatively easy, considering the isoforms are formed from the same gene.

Another potential avenue to explore would be to combine TipAct and FRET-MACF. This would result in a shortened MACF (containing only the ABD and the Microtubule Binding region) that is able to convey information about the force across it. By repeating the ‘’zippering” effect described in (López et al. 2014)[117],we could observe how this zippering would affect the forces across MACF. Subsequently probing whether this force behaviour is observed in vivo may tell us something about how MTs may move across actin in vivo. This may be quite different from the in vitro (minimal) experiments, as MACF may be affected by factors in vivo, and change behaviour due to binding sites becoming available as the molecule stretches.

As this research will uncover roles of Microtubule and actin crosstalk, it may be possible to use this interaction as a target for medication. If this crosstalk is essential for the survival, migration or transitions, it could have therapeutic potential. In metastasis, cells will have to cross various types of tissues, which is often accompanied by switching migration mode. We will only briefly explore the role of MACF in this process, but our platform research will hopefully allow facilitate further study. It will be easy to try metastasis inhibiting drugs for many different type of cancer cells in this setup. This way we can increase the rate at which drugs targeting such pathways are developed.

One critical thing that is missing at the time of this proposal is a list of the binding partners of MACF. As we mention, certain binding partners are known or being discovered.[124] If future research conducted an assay to discover all binding partners of MACF, results from this study could be explained (as we remove the regulatory region, but do not know all interactions in that section). New interactions could reveal novel ways of regulating cell migration. We believe a FRET construct of MACF will be very helpful if these regulatory interactions are characterised (to find whether they are force dependent or not).

Further research could also revolve around multiplex different fret sensors[127] of key migration proteins. This could reveal how such proteins interact in real time and how each respond to changes in the environment. Time delays in fret signals could, for example, reveal which protein is regulating the other. The research proposed here will be of use in such experiments, where the biggest obstacle would be the optics of such a setup.

5 Conclusion

In this research proposal, we have mentioned two novel research opportunities related to the general concept of cell migration. The proposed research would not only answer questions that are currently unanswered in this field, but also provide tools that can be used for follow up research, allowing for further exploration of the concepts we intend to address here.

Part I one of this proposal focuses on the design

and experiments related to a physical microfabricated setup for the study of the Amoeboid to Mesenchymal transition and its complement Mesenchymal to Amoeboid transition. This phenomenon is where cells switch from migrating in one fashion to another, where the movement types are classed into two distinct types (even though some research hints at more subtypes existing within these two distinct classes).

The platform proposed will be easy to fabricate initially using conventional lithography and microfabrication techniques. After generation of the master, it will be easy to fabricate many copies of the platform out of PDMS. This allows for easy adoption and high throughput in experiments. Due to the relatively simple fabrication process, it is also possible for labs to modify the specifics of the setup to their own needs. Changes in dimensions, exact geometry can all be altered, while still maintaining compatibility with any previously created imaging software or other aspects of the setup.

The study of the phenomenon of AMT/MAT can be valuable for cancer treatment, metastasis requiring AMT/MAT. Additionally, since each migrational mode is mechanistically distinct, observing the rearrangement of the molecular components during AMT/MAT will allow us to distinguish what is different between each mode in greater detail.

In Part 2, we addressed a large protein of the spectraplakin family. This protein is able to bind both the actin and microtubule cytoskeleton. As mentioned in the sections, this family has recently gathered some attention, and plays a significant role in cell migration, and in the interaction of microtubules in this context. As many molecules related to cell migration are already available in a FRET version, it would seem natural to expand this to a molecule that may serve as a linker between two cytoskeletal filament types that are both capable of generating forces.

By generating a FRET-MACF construct, we will be able to answer questions about the crosstalk between the different filaments in the cell, as well as investigate previously discovered interactions with a protein called ELMO. Combining Part 1 & 2, we will also study the crosstalk during AMT/MAT, hopefully opening up new avenues to explore regarding the interaction of microtubules and actin.

With these two aspects of cell migration, we hope that this research will be able to contribute not only to the current knowledge of migration, but also helps facilitate future research of this topic.

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